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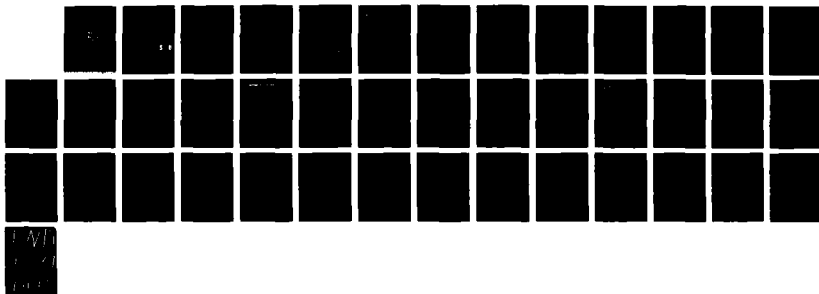
RAPID DIAGNOSIS OF ARBOVIRUS AND ARENAVIRUS INJECTIONS 1/1
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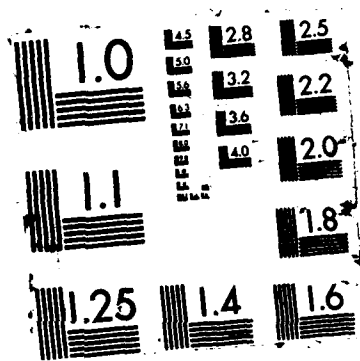
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**RAPID DIAGNOSIS OF ARBOVIRUS AND ARENAVIRUS INFECTIONS BY IMMUNOFLUORESCENCE
FINAL REPORT**

Gregory H. Tignor, Sc.D.

31 December 1984

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Yale University School of Medicine
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19. ABSTRACT (Continue on reverse if necessary and identify by block number) Spot-slides bearing cells infected with arboviruses were supplied to USAMRIID for immunofluorescence tests. A safe and quick ELISA for Crimean-Congo hemorrhagic fever (CCHF) virus was developed using infected, formalin-fixed CER cells as antigen. A retrospective serologic survey of equatorial Africa for antibodies in man to CCHF, Rift Valley fever (RVF), Ebola, Lassa fever, and Marburg viruses was done. In Sudan, antibodies to Marburg, CCHF, Lassa, RVF, and Ebola viruses were detected in prevalences between 0.2-11.2% with geographic clustering especially in the southern and central provinces. In Senegal, antibodies to Ebola, RVF, and Lassa were found. In Nigeria, antibodies to Lassa, Ebola, CCHF, and RVF viruses were recorded in prevalences from 5-14%. In Ethiopia, some sera from a putative yellow fever outbreak of 1960-62 were positive to Ebola virus. Ebola IgM was detected in one patient suggesting that Ebola was active in the 1960's. Keywords: arboviruses, arenaviruses,					
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SUMMARY

STUDIES ON RAPID DIAGNOSIS OF ARBOVIRUS INFECTIONS

Spot-slides bearing cells infected with arboviruses have been supplied to USAMRIID.

A safe and quick ELISA for CCHF virus was described. The basis of this test lies in the use of infected, formalin-fixed CER cells.

SUMMARY OF A SEROLOGIC SURVEY OF EQUATORIAL AFRICA

A retrospective serologic survey of equatorial Africa for antibodies in man to Crimean-hemorrhagic fever-Congo, Rift Valley fever, Ebola, Lassa fever, and Marburg viruses was done.

Sudan

587 sera collected in 1977-79 from Sudan were tested. The prevalence rates were Marburg 0.2%, CCHF 0.7%, Lassa 3.9%, RVF 4.6%, EBO-Zaire 8.2%, and EBO-Sudan 11.2%. Significant geographic clustering of antibody positive sera occurred for Lassa, EBO-Z and EBO-S viruses. For each virus, the northern provinces had little or no evidence of antibody, but the southern and southwestern provinces (bordering Central African Republic, Zaire, Chad and Uganda) had significantly higher rates (as high as 34% for EBO-S). The provinces located in central Sudan had varied prevalence rates. A village in one of the central provinces had a 26% rate for EBO (Z and S). However, villages within 100 miles had no positives.

Senegal

Ebola positive reactions were also found in sera collected in 1977 from life-long residents of Senegal's lower Fleuve region in the Senegal River Valley. Antibody to Rift Valley Fever and Lassa viruses was also found in Senegal.

Nigeria

Over 1000 sera collected in 1965/66 from the Benue River basin in Nigeria were screened on polyvalent slides. 985 were tested on monovalent slides. The greatest proportion of positives were to Ebola virus strains Zaire (6%), 14% positive to CCHF virus, 9.8% positive to Lassa virus, and 5% positive to RVF virus.

Ethiopia

A retrospective study of sera collected during the yellow fever epidemic in Ethiopia, 1960-1961 was conducted. In the epidemic region, we found that 22% of 50 sera were positive while in some sub-regions, positives ranged from as few as 10% to as many as 38%. These results suggest that there was a concurrent outbreak of Ebola infections. Confirmation of this hypothesis was obtained when IgG antibody was found in 4 of 5 ill patients who had no group B virus antibody. The fifth patient in this group had IgM antibody for Ebola virus suggesting a recent infection.

FOREWORD

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).



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Results during the entire period of the contract (1 August 1977 to 31 December 1984). The present principal investigator served since August, 1981.

The working hypothesis made at the outset of this project was that an early diagnosis of infection by arboviruses and arenaviruses could be made in a simple and practical manner by means of the IF test. The hypothesis was to be tested by applying the test to the detection of antigen, virological diagnosis, and of antibody, serological diagnosis.

As it was conjectured that virus-containing specimens from current cases would be hard to come by, the feasibility of rapid recognition of antigen was to be tested either by producing an infection in small laboratory animals and testing their early sera for viremia; or by using viral stocks as a substitute for clinical specimens as a source of pathogens.

The realization that early diagnosis of certain arbovirus diseases -- CCHF, RVF -- and of diseases caused by viruses designated as "special pathogens" -- Lassa, Marburg, Ebola, KHF -- could be improved by previous knowledge of the world distribution of these agents, stimulated an additional phase of research, seroepidemiological surveys. The surveys were initiated using as antigens in the IF test cells from infected tissue cultures deposited on microscope slides, fixed with acetone (spot-slides) and stored at low temperature.

MATERIALS AND METHODS

Viruses. The viruses and strains used were: Banzi, SA H336; Congo-Crimean hemorrhagic fever, IbAr10200; Japanese encephalitis, Nakayama; Langat, TP21; Pongola, MP 781; Rocio, San Paulo; Sicilian phlebotomus fever, Sabin; West Nile, Egl01; yellow fever, Asibi. the viruses are maintained as 10% infected newborn mouse brain tissue suspensions held lyophilized or wet frozen at -60°C.

Immune reagents. Reference reagents available at the World Reference Center, YARU, were used, including polyvalent grouping and monovalent reagents. These reagents were mouse sera and ascitic fluids and sera from hamster, guinea pig and rabbit, kept wet frozen at -20°C or lyophilized at 4°C.

Cell cultures. The following cells routinely maintained in this laboratory have been used: VERO, BHK-21, LLC-MK2 and CER. The cells were maintained in 150 cm² plastic bottles; transfers were made every 8-10 days for VERO and LLC-MK2, every 4-6 days for the other cells. Fresh cultures were started from cells held in liquid nitrogen when it seemed warranted.

Animals. The following have been used: mouse (Mus musculus), Swiss outbred; hamster (Mesocricetus auratus); guinea pig (Cavia porcellus); and rabbit (Oryctolagus cuniculus). The animals were purchased from commercial dealers; mice were used as newborn, 1 to 5 days old, or as adults, as required.

Bleeding of all animals and intracerebral inoculations were done under deep ether anesthesia. Whole bloods or sera to be tested for infective virus were stored at -60°C ; sera for antibody tests, at -20°C .

PREPARATION OF SPOT-SLIDES.

In essence the method used, described in full detail before in the Second Annual Report, January 1979, was as follows.

Cell cultures in 150 cm^2 plastic bottles showing a complete monolayer were infected with a virus at an MOI between 1 and 100. When CPE was 1+ or 2+, or after a predetermined number of days with viruses that replicate with no CPE, the cells were harvested, dispersed with trypsin and versene (no versene was used with BHK-21 and CER cells), washed with phosphate buffered saline (PBS) and generally adjusted to a 3×10^6 cells/ml count; similar suspensions were prepared with uninfected cells. The two suspensions were mixed in a proportion of 3 or 2 parts infected to 1 of uninfected cells; the cell suspension mixture in volumes of 0.01 ml (or 3×10^4 cells) was dropped on the surface of teflon-coated slides (Cel Line Associates, Minotola, N.J.) which have 12 circular areas 5 mm in diameter. The drops were allowed to dry by holding the slides at 37°C for 30 minutes; the slides were next immersed in acetone for 10-12 minutes and stored at -60°C . Slides have been used nearly 2 years after preparation with excellent results.

For polyvalent slides, individual suspensions were prepared as described above with each of the viruses; a mixture of equal parts of each infected suspension and of a non-infected suspension of one of the cell lines was made and used.

Preparation of chamber slides. Tissue culture slides 75 x 25 mm with 4 chambers were used (Lab-Tek Products, Miles Laboratories, Naperville, Illinois). Monolayers under fluid medium were prepared by seeding each chamber with 2 to 2.5×10^5 cells in a volume of 0.8 or 0.9 ml of growth medium; the slides were held in a CO_2 incubator. One to three days later the monolayers were infected with the virus, or with a virus-serum mixture for a neutralization test, in a 0.1 ml volume; maintenance medium was added after a period of adsorption of 1 h and the slides again held at 37°C in the CO_2 incubator. At the desired time, 1 to 5 or 6 days later, the chambers were drained and removed and the slides fixed in acetone for IF staining.

IMMUNOFLUORESCENCE TEST.

Isothiocyanate conjugates were purchased from commercial dealers; preparations in routine use were conjugated anti-globulins for man, mouse, hamster, guinea pig and rabbit. The indirect technique used was a standard laboratory procedure (Gardner and McQuillin, 1975); Evans' blue at a final dilution of 1:10,000 was used as a counter-stain. An Olympus "Vanox" incident light microscope set for blue fluorescence and a mercury burner light source were used. When viewed with x40 and x100 immersion objectives, glycerol directly over the slide without a cover-slip was used.

For routine testing, human sera were screened at dilution 1:4; positive sera were titrated at increasing two-fold dilutions. On occasion sera were tested undiluted and at dilution 1:2; in these instances and also when it was deemed necessary to ascertain whether weak reactions were truly specific IF or the result of adsorption of dye by precipitates in the serum, the sera were extracted with acetone and ethyl ether (Casals and Tignor, 1974).

The results of the IF tests are given in this report according to the following rating. Sera tested only at dilution 1:4 are rated "strongly positive" when the brightness or intensity of fluorescence was 3+ or 4+, in a scale from 0 to 4+; "medium positive", when it was 2+; and "weak positive", when it was 1+. Sera positive at dilution 1:8 or higher are all rated "strongly positive".

Other serological tests. Other tests used, CF, HI and neutralization tests in mice or cell cultures have been described in detail (Casals, 1967; Schmidt, 1979). The CF and HI tests were semi-micromethods with a total volume of reagents of 0.15 ml (6 drops) and 0.1 ml (4 drops) respectively.

Human sera. As far as could be ascertained all sera for serological surveys were obtained from seemingly well individuals. The sera were stored wet frozen at -20°C, with the exception of the sera from Ethiopia which had been lyophilized at the Pasteur Institute, Addis Ababa, at the time of collection and subsequently stored at -20°C.

RESULTS

SPOT-SLIDES FOR IF TESTS SUPPLIED TO USAMRIID.

As part of the contract, spot-slides were prepared, tested for specificity and adequacy and shipped to USAMRIID; two ampules of virus stocks and 6 x 0.5 ml of hyperimmune mouse serum or ascitic fluids were included in the shipments. The viruses and cells used and the number of slides supplied were:

Polyvalent group B, Banzi, Japanese encephalitis, Langat,
Rocio and yellow fever; several cells; 200

Rocio, San Paulo, VERO; 200

Yellow fever, Asibi, VERO; 200

Dengue type 4, strain H 241; 277, September, 1981

Polyvalent group B, tick-borne; 220

Group B polyvalent, mosquitoes; 280

Dengue type 4, strain H 241; 260

Rocio (H34675); 106

Yellow fever (Asibi); 220

yellow fever (asibi); 100
 Sicilian SFF, CER; 417
 West Nile, Vero; 220
 Oropouche, Strain Tr9760, Vero, 220
 Group B, polyvalent, Vero, LLCmk2, BHK 21; 300
 Dengue type 4, H 241, LLCMK2, 216
 Dengue type 2, NGB, LLCMK2, 216, December, 1978
 Dengue type 2, NGB, LLCMK2, 216, October, 1978
 Yellow fever (Asibi), Vero, 216, September, 1978,
 Chikungunya (Ross), Vero, 216, August, 1978
 Naples SFF, (Sabin Strain), 218, April, 1978
 EEE (Alabama Strain), 219, March, 1978
 Langat (TP21), 220, March, 1978
 LCM (Bulgaria), 200, January, 1978
 LCM (Bulgaria), 226, February, 1978
 JE (Nakayama), Vero, December, 1977
 Dengue type 1, (Hawaii), LLCMK2, November, 1977
 VEE, TC80), Vero, October, 1977
 Dengue type 1 (Hawaii), LLCMK2, September, 1977

INACTIVATION OF SPOT-SLIDES.

CCHF virus spot-slides that had been stored at -60°C for 90 days were irradiated with ultraviolet light (UVL) from the burner in a Baker Biogard hood, the distance between burner and slides being 45 cm; a set of slides was irradiated for 10 minutes, another for 20 minutes and a third was not irradiated. Irradiated and non-irradiated slides were tested for virus; drops of diluent were deposited on the spots and the tissue scraped off the glass. The total volume of diluent used for each slide (10 spots) was 0.5 ml; this suspension was called "undiluted". The 3 suspensions, 10 and 20 minutes irradiated and non-irradiated, were diluted to 10^{-3} and intracerebrally inoculated to 3-day-old mice in groups of 8 mice per dilution. No mice died or showed signs of illness, including those inoculated with non-irradiated slides;

in this respect the experiment failed to show whether UVL had inactivated the virus. On the other hand, the experiment showed that the method used for preparation of slides --heating at 37°C for 30 minutes and immersion in acetone for 10-12 minutes --followed by 3 months storage at 60°C resulted in virus inactivation. No difference was observed between irradiated and non-irradiated slides when an immune mouse serum was titrated with them; the intensity of fluorescence, location of fluorescent antigen in the cells and titer of the serum were the same in the 3 sets.

Yellow fever, Asibi strain, spot-slides were inactivated by immersion in betapropiolactone (BPL) at dilution 0.1% in PBS, pH 7.2; following this treatment, the slides were dried and stored at -60°C until tested for virus and in the IF test. Slides, treated with BPL and untreated, were tested for virulence as described in the preceding paragraph and for efficacy in the IF test by titration of an immune mouse serum. Two attempts were carried out with slides that had been stored 4 and 5 months, respectively, at -60°C after preparation; the result of the intracerebral titrations in newborn mice is shown in Table 2.

Following 10 minutes inactivation in experiment #1 there was possibly some residual virus; in test #2, the deaths are considered non-specific as they occurred 10-12 days after mouse inoculation. In the IF test, the slides in experiment #1 after 10 minutes exposure to BPL, were nearly as good as the untreated ones. In experiment #2 all treated slides regardless of length of treatment were alike and definitely inferior to the controls. The IF seemed bleached out and hard to read at x20, somewhat better at x 40 magnification; however, the titer of the serum was the same against the 3 sets of slides.

APPLICATION OF IF TO VIRUS IDENTIFICATION.

The IF test was employed on several occasions as a practical rapid step for identification of viral isolates. A strain from Fiji, #41451, from a febrile patient, was inoculated into BHK-21, VERO, CER, Aedes albopictus and A. pseudoscutellaris cell cultures; spot-slides were prepared with the cells and tested against grouping polyvalent mouse immune ascitic fluids for groups A, B and an additional 10, with the result that only group A fluid gave a positive reaction with the slides. The isolate was ultimately identified as Ross River virus.

A similar procedure was followed with 2 strains isolated in Uganda, Ug MP-15332 and UgZ-52969 and one in South Africa, SA An-24630. Cell cultures BHK-21, VERO and CER were inoculated with the strains, spot-slides prepared and tested with polyvalent grouping reagents. Isolate SA An - 24630 reacted only with the group B fluid; Ug MP - 15332 only with Bwamba; and UgZ - 52969 with none of 28 grouping fluids. In the course of time, SA An - 24630 was identified as a strain of West Nile, Ug MP - 15332 as Pongola and Ug Z - 52969 as a new virus distantly related to Yogue virus.

In a converse procedure, an immune mouse serum prepared against a strain, CS-122, submitted from Australia, was tested with polyvalent groups A and B slides; the serum reacted only with the latter. The virus was finally identified as a new group B agent.

APPLICATION OF IF TO RAPID IDENTIFICATION OF CCHF VIRUS.

In the Annual Report for the 3rd period, January 1980, it was shown that rapid identification of JE, Banzi and Junin viruses could be done by daily processing for IF of cell monolayers in chamber-slides inoculated with the viruses.

A similar study was carried out with CCHF virus. A virus stock, strain Ib Ar 10200, having an LD₅₀ of $10^{-6.5}/0.02$ ml by intracerebral inoculation to 2-day-old mice was used in dilutions 10^{-3} , 10^{-4} , and 10^{-5} . Monolayers of CER cells were prepared in 4-chambered slides by seeding 2×10^5 cells in 0.9 ml of medium in each chamber; 24 hours later, 4 slides were inoculated with 0.1 ml of each virus dilution, the 4th chamber receiving diluent instead of virus. No CPE was noted during the duration of the experiment. A slide was examined in the IF test on days 1, 2, 3 and 5 after inoculation of the virus; as source of antibody was used an anti-CCHF serum, having a titer of 1:512, at dilution 1:10. The result of the tests is shown in Table 3.

As shown in Table 3, the number of ICLD₅₀ doses contained in 0.1 ml of each dilution was, respectively, 1.5×10^4 , 1.5×10^3 and 1.5×10^2 . The IF reaction was strongly positive with all the combinations of time and dilutions, with a 4+ or maximum fluorescence in all instances except a 3+ with the smallest inoculum (10^{-5}) containing only 300 LD₅₀, tested 1 day after infection.

APPLICATION OF IF TO DIAGNOSIS OF KOREAN HEMORRHAGIC FEVER.

Diagnosis of KHF has been made possible by the propagation of the etiological agent in cell cultures and its detection by IF (Lee et al., 1978;

Lee *et al.*, 1980). Spot-slides bearing suspensions of cells of human carcinoma A-549, infected with KHF were supplied by Dr. G. French, USAMRIID; control, uninfected slides were also supplied. Three sets of available sera were tested in an attempt to determine the suitability of these slides as diagnostic antigens: a) Fifteen convalescent sera from persons admitted to the Hospital of the Hubei Provincial Medical College, Wuchang, China, between 1975 and 1979 with the clinical diagnosis of epidemic hemorrhagic fever (Cohen *et al.*, in preparation, 1981); the sera were supplied by Professor C.M. Hsiang, of that Medical School. b) Ten paired sera from patients admitted to 3 hospitals in South Korea in 1974, clinically diagnosed as KHF; the sera were supplied by Dr. K. H. Kim, National Institute of Health, Seoul, Korea. c) Single sera taken during convalescence from 10 U.S. Army personnel, with a clinical diagnosis of KHF in 1967-68; the sera were given by Dr. Ned H. Wiebenga, Chief, Epidemiology Branch, Department of Health, Hawaii.

The sera were tested by IF at dilution 1:4 only, with the exception of two that were titrated in increasing 4-fold dilutions. All 15 sera in group a) gave positive reactions with readings of 3+ or 4+, except one serum that gave a questionable reaction; the reactions with the control slides was uniformly negative with these as well as with the remaining sera. The results with the sera in groups b) and c) are given in Table 4. Intense positive reactions were given by all patients except KHF-5, which may have been a false clinical diagnosis. Acute sera taken as early as 6 days from onset were strongly positive; titrations of two sera, J.H.L. and J.D.Y., resulted in titers of 1:2048 and 1:512, respectively.

SEROLOGIC STUDIES WITH KHF VIRUS.

E.M. studies with KHF virus suggested that KHF virus might be a Bunyavirus-like particle. Subsequently, spot-slides of KHF-infected cells were prepared and delivered by Dr. R. Rosato to Dr. G. Tignor by instruction of Dr. K. Johnson for IF screening using antibody to all known members of the Bunyamwera supergroup. In our tests, most members were tested by FA or complement fixation against their homologous sera or ascitic fluids. Grouping sera or ascitic fluids were not used in these tests by instruction of Dr. K. Johnson. A summary of our results is presented below.

TESTED	POSITIVE	QUESTIONABLE	NOT TESTED
158	4	18	43

All of the questionable reactions have been eliminated as tissue reactions since these sera reacted with uninfected cells. One positive reaction which we report is with Manawa virus (strain JC 791) antibody made with infected mouse brain tissue on KHF (Rosato) slides. Only one of several antibody preparations to Manawa virus reacted positively. It may be that this particular strain of Manawa virus contains more than one agent. The hypothesis is supported by the fact that our other positive reactions occur within one virus group. The other positive reactions were to Bahig, Matruh, Tete, all group Tete viruses. Antibody to these three viruses also reacted positively on KHF (Lee) slides. We determined that these reactions were tissue reactions seen with only some sets of KHF infected cells. Normal vero cells from selected sources reacted with

these sera. Other vero cells did not. A complete summary of our test results follows.

Human immune sera (from Wiebenga) were tested in parallel on KHF slides from Lee and Rosato. The following reactions were observed.

SERUM #	OLD KHF SLIDES		NEW KHF SLIDES (ROSATO)	
	Acute	Convalesc	Acute	Convalesc
KHF 1*	++	++	+	++
KHF 2*	++	++	NT	+
KHF 3*	++	++	++	NT
KHF 4*	++	++	NT	++
KHF 5	0	0	0	0
KHF 6	++	++	+	++
KHF 7	++	++	NT	++
KHF 8	++	++	++	++
KHF 9	++	++	++	++
KHF 10	++	++	++	++
Loomis	NT	++	NT	++

NT was not tested; * was negative with uninfected cells

The detailed cumulative results of our tests are presented below. A positive reaction is indicated by (+) and a negative by (0). Tests which were inconclusive were repeated and these results are indicated by (-). Most of these reagents also reacted with uninfected cells.

IF REACTIONS WITH KHF SPOT SLIDES

IMMUNE REAGENT	RESULT	IMMUNE REAGENT	RESULT
Anopheles A		C Group contd.	
Anopheles (CoAr 3624)	NT	Madrid	0
Lukuni	0	Marituba	0
ColAn 57389	-	Murutucu (BeAn 974)	NT
Tacaiuma (BeAn 73)	0	Restan	0
		Nepuyo	0
Bunyamwera Group		Oriboca	NT
Bunyamwera	0	Itaqui	-
Germiston	0	Gumbo Limbo	0
Shokwe	NT		
Batai (Calovo)	-	California Group	
Birao	0	California	0
Tensaw	0	Tahyna	0
Cache Valley	0	Inkoo	0
Maguari	-	San Angelo	0
Northway	0	La Crosse	NT
Santa Rosa	0	Melao	0
Lokern	-	Serra do Navio	NT
Wyeomyia	0	Keystone	NT
Taiassui	NT	Jamestown Canyon	0
Anhembi	0	Trivittatus	0
Sororoca	0		
Main Drain	0	Capim Group	
Kairi	0	Capim	0
Guaroa	0	Guajara	0
76V-25880	0	Bush Bush	NT
Macaua	0	BeAn 84381	-
ACRE	0	Gu 71 u 344	NT
Mojui Dos Campos	0	Juan Diaz	0
AG 80-381	0	Acara	0
AG 80-504	0	Moriche	NT
PARA	0	BeAn 153564	NT
Virgin River	0		
		Guama Group	
Bwamba Group		Guama	0
Bwamba	0	Moju	0
Pongola	0	BeAn 109303	NT
		Mahogany Hammock	0
C Group		Bimiti	0
Caraparu (BeAn 3994)	-	BeAn 116382	0
Caraparu (BeH 5546)	NT	Catu	0
Caraparu (Trinidad)	NT	Bertioga	0
Ossa	-		
Apeu	-		

IMMUNE REAGENT	RESULT	IMMUNE REAGENT	RESULT
Koongol Group		Tete Group	
Koongol	0	Bahig (EgB 90)	+
Wongal	NT	Matruh (EgAn 1047-61)	+
Mirim Group		Truruse	NT
Mirim	0	Batama	NT
Minatitlan	0	Tete (EgAn 4511)	+
Olifantsvlei Group		Anopheles B	
Olifantsvlei	0	Anopheles B	-
Bobia	NT	Boraceia	0
Botambi	0	Bakau Group	
Patois Group		Bakau	0
Patois	0	Ketapang	0
Group		Crimean hemorrhagic Fever	
Shark River	NT	Congo	0
Zegla	0	Hazara	0
Pahayokee	NT	Kaisodi Group	
Simbu Group		Kaisodi	0
Akabane	0	Lanjan	0
Yaba-7	0	Silverwater	0
Facey's Paddock	NT	Mapputta Group	
Shamonda	NT	Mapputta	0
Sango	0	Maprik	NT
Sabo	NT	Gangan	NT
Sathuperi	0	Trubanaman	NT
Shuni	0	Nairobi Sheep Disease Group	
Aino	0	Ganjan	0
Simbu	0	Dugbe	-
Thimiri	0	Phlebotomus Fever Group	
Nola	0	Candiru	0
Peaton	NT	Itaituba	0
Manzanilla	0	Nique	0
Ingwavuma	0	Punto Toro	0
Mermet	0	Buenaventura	-
Inini	0	Saint Floris	0
Buttonwillow	0		
Oropouche	0		
Utinga	0		

IMMUNE REAGENT RESULT

Phlebotomus Fever Group cont.

Goril	-
Aqucate	0
Anhanga	0
Arumowot	0
Bujaru	0
Cacao	0
Caimito	0
Chagres	0
Chilibre	0
Frijoles	0
Icoaraci	0
Itaporanga	0
Karimabad	0
Pacui	0
Rio Grande	0
Salehabad	-
Gabek Forest	NT
SF Naples	0
Toscana	0
SF Sicilian	0
Urueuri	0
Alenquer	NT
Tehran	0
Joa	0
Muwguba	0
Orixima	0
Turuna	-
Rift Valley Fever	0
Belterra	0

Sakhalin Group

Sakhalin	NT
Tillamook	NT
Taggert	NT
CloMor	0
Avalon	-

Thogoto Group

Thogoto	0
SiAr 126	NT

Turlock Group

Turlock	0
Umbre	0
M'Poko	NT
Barnah Forest	NT
Marweh	NT

IMMUNE REAGENT

RESULT

Uukuniemi Group

Uukuniemi	0
Ocean Side	0
Grand Abaud	0
Manawa	+
Zalin Terpeniya	-
Ponteves	NT
Egan 1825-61	-

Unassigned Viruses

Belmont	0
Bhanja	0
Khasan	NT
Kowanyama	0
Lone Star	0
Razdan	NT
Rift Valley Fever	0
Sudany Canyon	NT
Tamdy	NT
Tataquine	NT
Witwatersrand	0
Gamboa	0
Guaratuba	0
Kaeng Khai	0

**ANTIBODIES AGAINST YELLOW FEVER VIRUS AFTER VACCINATION WITH 17D:
COMPARISON OF NEUTRALIZATION, HI, IF AND ELISA TESTS.**

Antigen for ELISA test was prepared by precipitation of the virus grown in cell culture by polyethylene glycol and centrifugation in a discontinuous sucrose gradient. The result of the test was recorded with an ELISA-reader (Titertek) and the titer of a serum was determined by comparing its regression analysis slope with that of a control serum. Pre- and post-vaccination sera from 13 individuals were compared by plaque reduction neutralization test at 90% and 50% reduction, HI, IF and ELISA, with the results shown in Table 5. The conclusion of this investigation was that following vaccination with 17-D yellow fever vaccine, neither ELISA nor IF tests detect antibodies, with few low titer exceptions, in persons who were positive both by plaque reduction and HI tests. Two of the 13 individuals who were strongly positive by ELISA after vaccination were also positive with the same titers before vaccination; the reaction was non-specific, as the plaque reduction and HI tests show.

SEROLOGICAL SURVEYS BY IF TEST.

Human sera from Ghana, Ethiopia, Sudan, Senegal, Cameroon, Liberia, Greece, Pakistan and United States were variously tested for antibodies against Ebola, Lassa, Marburg, KHF and CCHF viruses. The results will be reported in detail in the following sections.

Cameroon. Sera were collected in that country as part of a survey for antibodies against Lassa fever virus in equatorial and West Africa (see later, under Liberia). Forty-two sera originating in Garoua-Boulai have been tested with ELM and Lassa slides; positive reactions were observed only with ELM slides, 3 strong and 3 medium; a serum had a titer of 1:32, another a titer of 1:8. It is tentatively concluded that these antibodies are directed against Ebola rather than Marburg virus.

Liberia. A large scale survey for anti-Lassa virus antibodies in several countries of equatorial and West Africa was in operation for several years, in association with Dr. J.D. Frame, College of Physicians and Surgeons, Columbia University, New York. A total of 311 sera collected in 1978 and 1980 in Liberia, mainly in the areas of Foya and Zorzor, were tested against Lassa slides only; 32 sera were strongly positive and 19 medium positive, with 3 having titers in the range 1:256 and 1:512.

Greece. Sera were collected in northern Greece by Dr. A. Antoniadis, Aristotelian University of Thessaloniki, School of Medicine and tested by him at YARU. A total of 323 sera were tested by IF against CCHF virus and 29 against KHF virus; with the exception of 3 or 4 sera that gave questionably positive reactions against the former, all results were negative.

Pakistan. About 200 human sera collected in that country in 1978-79 were supplied by Dr. C. Hayes, Gorgas Memorial Laboratory, Panama; the sera are intended for a survey for CCHF and KHF virus antibodies. In the course of 1980, 49 sera were tested against CCHF and 24 against KHF, all with negative results.

United States. The fact that so many human sera from certain areas of Africa had antibodies that reacted with ELM slides in the absence of recognized

disease caused by Ebola, Marburg and Lassa viruses, made it desirable to test sera from a contrasting population as a control for specificity of the reaction. To this end, sera from 35 residents in two areas of the United States, northeastern and Alabama, were included in tests with polyvalent ELM slides; all these sera were negative in blind tests.

SEROLOGICAL SURVEY IN THE SUDAN.

(In collaboration with J. Meegan and T. Bucci, NAMRU-3).

It is likely that many viruses which circulate in northern and southern Africa are disease problems in Sudan. Few studies have been undertaken in this geopolitically important country. During 1979, 1980, and 1981, over 3400 sera were collected from military recruits in Sudan. These represent collections from all areas of Sudan (age, birthplace, and district of residence are available for all). This survey was one phase of a long-term study to determine the impact of a number of virus infections on humans and animals in Sudan. In addition, since Sudan may act as a tunnel for the movement of viral disease from sub-Saharan Africa to Egypt and beyond, survey for other viruses may give clues as to what diseases to be alert for.

Twenty to forty sera from soldiers native to villages near each base where sera were collected were tested on polyvalent CRE2LM slides. Since these soldiers grew up and were stationed in the same area, we used them as an indication of which areas in Sudan were endemic for hemorrhagic fever viruses. Interestingly, only one sera of 55 collected in area near Khartoum was positive (for RVF virus) while rates of greater than 25% were seen in southern provinces. Since soldiers stationed in and around Khartoum had little chance of contracting the disease during military training, we considered sera from these soldiers are representative of the antibody prevalence rates in their native governorates, and included them in our studies of the distribution of these viruses in Sudan. Over 580 sera were screened on polyvalent CRE2LM slides and most retested on monovalent slides. Table 1 gives the prevalence data for antibodies to each virus (refer to Figure 1). Significant geographic clustering of antibody positive sera occurred for Lassa, EBO-Z and EBO-S viruses. For these viruses, the northern provinces had little or no evidence of antibody, but the southern and southwestern provinces (bordering Central African Republic, Zaire, Chad and Uganda) has significantly higher rates. The provinces located in central Sudan had varied prevalence rates. The majority of endemic areas have a savanna type of vegetation; the major economic activity is grazing (Figures 2 and 3).

In the central province of Southern Kordofan, one village (Muglad) had a high antibody prevalence rate for Ebola virus (Table 2). But villages in the same ecological zone within 100 miles (Figure 4) of Muglad showed low antibody prevalence rates.

Figure 1
Study Region in the Sudan

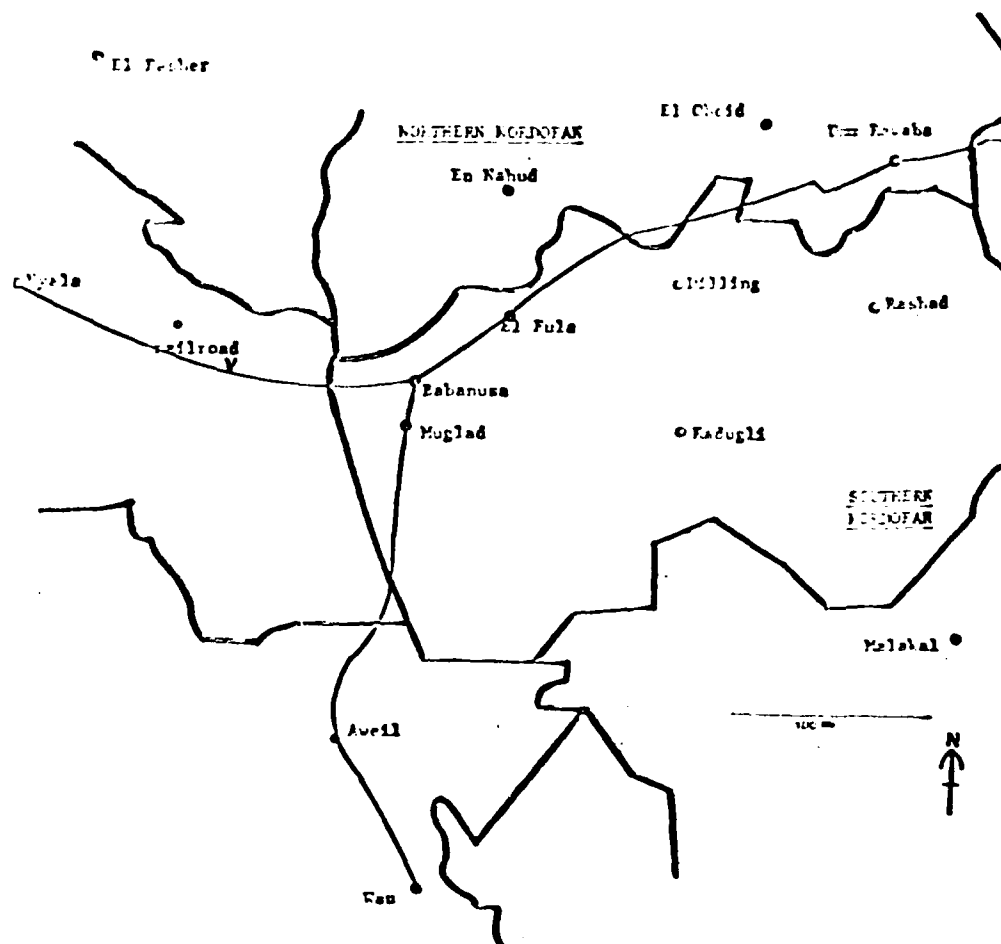


Table 1

Sudan sera seropositive in immunofluorescence tests using polyvalent antigen slides containing Ebola, Marburg, Lassa, Crimean-Congo, and Rift Valley fever viruses.

Source of Sera (Home of Governorates)	Governorate where currently stationed	Number Positive/ Number tested	Percent
Northern Governorates (Nile, Northern, Kassala, Khartoum, El Gezeria, White Nile)	Khartoum	1/55	1.8
Southern Governorates			
Eastern Equatoria	Eastern Equatoria	7/34	26.6
Western Equatoria	Western Equatoria	18/47	38.3
Bahr El Ghazal	Bahr El Ghazal	33/59	56.0
Central Governorates			
Northern and Southern Kordofan	Khartoum	2/56	3.6
Northern and Southern Kordofan	Eastern and Western Equatoria	9/56	16.1

Table 2

Summary of Sudan Positives

		Percent Positive					
Province	Number sera tested	RVF	CCHF	EB0-Z	EB0-S	Lassa	NAR
1. E. Equatoria	35	5.7	2.9	17.1	20.0	8.6	0.0
2. W. Equatoria	51	5.9	0.0	25.5	21.6	2.0	7.0
3. El Fuleyrat	(8)	(12.5)	(0.0)	(0.0)	(12.5)	(12.5)	(0.0)
4. Junglei	30	13.3	0.0	0.0	10.0	0.0	0.0
5. Bahur El Ghazal	62	8.1	1.6	16.1	27.4	16.1	0.0
6. S. Darfur	33	3.0	0.0	0.0	0.0	0.0	0.0
7. S. Kordofan							
Muglad area	34	8.8	0.0	7.9	26.5	0.0	0.0
non-Muglad area	38	2.6	0.0	0.0	0.0	2.9	0.0
8. Upper Nile	10	10.0	0.0	0.0	0.0	0.0	0.0
9. N. Darfur	33	0.0	0.0	0.0	3.0	0.0	0.0
10. N. Kordofan	31	0.0	0.0	3.2	0.0	3.2	0.0
11. White Nile	1	-	-	-	-	-	-
12. Blue Nile	6	-	-	-	-	-	-
13. El Gezira	15	0.0	0.0	0.0	0.0	0.0	0.0
14. Farsala	31	3.2	0.0	3.2	0.0	0.0	0.0
15. Khartoum	25	4.0	0.0	0.0	0.0	0.0	0.0
16. Nile	16	0.0	0.0	0.0	0.0	0.0	0.0
17. Red Sea	19	0.0	0.0	0.0	15.8	0.0	0.0
18. Northern	20	5.0	0.0	0.0	0.0	0.0	0.0

Table 3

Sudan Positives by Region

<u>Village</u>	<u>Number sera tested</u>	<u>Number positive (LEO-2 and LEO-5)</u>
Muglad	34	10
Dilling	12	0
Fadugli	7	0
El Fula	8	0
El Obeid	18	1
El Fasher	30	1
Nyala	29	0
Wau	50	16

**SENEGAL HUMAN SEROSURVEY FOR RIFT VALLEY FEVER, EBOLA, MARBURG, LASSA, AND
CRIMEAN-CONGO HEMORRHAGIC FEVER**

A serological survey of human sera collected in Senegal by a YARU team headed by W.G. Downs in 1977 was done using indirect immunofluorescence and spot-slides. The slides were provided by Dr. K.M. Johnson, CDC. Polyvalent slides were used to screen 283 sera for RVF, Ebola, Marburg, Lassa, and CCHF. Not all sera were tested for all viruses. Of 283 sera tested, 37 were positive to one or more antigens.

Six sera were positive with spot-slides containing all 5 viruses; 4 out of 5 of those tested for RVF were mono-specific for RVF. Only 4 of 31 positive to the polyvalent Ebola-Marburg-Lassa spot-slides were tested further.

The cumulative positive results indicate Ebola antibody in 6 persons (2 were children aged 5 and 8 years), Lassa antibody in one person, and RVF antibody in 4 persons. All of these persons claimed to be life-long residents of Senegal's lower Fleuve region in the Senegal River valley. This is the site where multi-national construction teams are about to start building a series of dams.

In view of the surprising finding of RVF antibody and the know cross-reaction by IFA with other phlebotomus fever group viruses, tests were done to determine the specificity of the reaction with 2 of the sera. The results indicated full specificity for RVF. In addition, PRNT performed by Dr. C.J. Peters, USAMRIID, confirmed the RVF positive reactions.

SEROSURVEY IN GHANA

Two hundred forty-seven human sera collected in Ghana in 1975 were screened on ELM slides. Many positives were found with sera from individuals in the Eastern (42%) and Western (34%) regions and fewer positives from the Ashanti (18%) region.

Ghana positives on ELM slides by geographic region

Region	Results		
	Positive	Tested	Percent
Eastern	35	83	42
Western	35	102	34
Ashanti	11	62	18

31 that were positive were subsequently tested with monovalent Ebola, Lassa and Marburg slides. In addition, 47 sera were tested for antibodies against CCHF virus. 37 sera were positive. The titers of 7 sera titrated were; 1:32, 3 sera; 1:16, 3 sera; and 1:8, one serum. Tests with monovalent slides were positive only for Ebola; 5 sera that had been positive with polyvalent ELM sides, failed to react positively with any of the monovalent slides. It is conceivable that the individual antigens used in the polyvalent slides were more potent than those used for the monovalent slides. It should be pointed out that those sera

were collected about one year before the disease, Ebola hemorrhagic fever, was observed for the first recorded time.

SEROSURVEY IN ETHIOPIA

We have screened 201 sera from the Ethiopian collection taken during the 1962-63 yellow fever epidemic.

BACKGROUND AND HYPOTHESIS.

The epidemiologic features of the Ethiopian yellow fever epidemic during 1960-1961 were described by Serie et al. The epidemic began with cases of YF which were seen in 1959 in Sudan. A survey in Wollenga province demonstrated 200 cases with 100 deaths. In December, 1960, in Gamu Goffa province, there were 3000-8000 deaths in a population ranging from 50,000 to 80,000. There was an 18 month epidemic in south-east Ethiopia, with 30,000 mortality in a population of 1,000,000. Morbidity was estimated at approximately 100,000. The epidemic was rural in character; adults were more frequently affected than children and men more than women. Serological conversion occurred after the epidemic, suggesting, to the authors, that the virus continued to circulate, "apparently in greatly attenuated form."

The clinical cases took two distinctly different forms. The first was a diphasic illness separated by a brief period of remission. The second and more fulminating form was characterized by intense general symptoms and rapid death, usually within 2-3 days. The explosive nature of this epidemic destroyed the clinical impression that Blacks do not die as frequently of yellow fever as non-blacks. This epidemic stood in contrast to all previous epidemics of yellow fever recorded in Africa with the exception of a very early incompletely reported epidemic in the Sudan. One opinion was that there was no preexisting group B antibody which served to modify the disease in this area. However, the data obtained during the course of the epidemic argued against this possibility.

In 1962-63 a large number of sera were collected in that country by Dr. C. Serie, at the time director of the Pasteur Institute, Addis Ababa, of which around 400 were submitted to the Rockefeller Foundation Virus Laboratory, the predecessor of YARU; thus far, 115 sera originating mainly in the valleys of the Awash and Blue Nile rivers have been tested against Ebola, Lassa and Marburg viruses. Only positives with Ebola virus were found with the monovalent slides. One serum gave a titer of 1:128, 2 sera titers of 1:32 or higher, and another 2 sera had titers of 1:16. These results show that antibodies capable of reacting with Ebola antigen were present in the population of Ethiopia 13 or 14 years before the disease was first recognized.

Ethiopian survey results by region on ELM slides

Region	Positive	Negative	Per Cent
Assab	3	17	15
Blue Nile	26	44	37
Sidamo-Borena	10	50	17
Ogaden	0	4	0
L'Aquache	14	33	30

Table 4

SUMMARY OF SELECTED HI RESULTS DURING ETHIOPIAN YF EPIDEMIC

KAFFA PROVINCE

GROUP B	49%
GROUP A	13%
YF	26%
ZIKA	7%
NON-DIAGNOSTIC	11%

Table 5

SUMMARY OF SELECTED HI RESULTS DURING ETHIOPIAN YF EPIDEMIC

VILLAGE OF GORA

TESTED	39
GROUP B	29 (74%)
YF	1 (6%)

Table 6

SUMMARY OF SELECTED HI RESULTS DURING ETHIOPIAN YF EPIDEMIC

VILLAGE OF TCHABORA AGORE

TESTED	75
YF	28 (37%)
ILL PATIENTS	18
" " WITHOUT B ANTIBODY	5 (28%)

Table 7

SUMMARY OF EBOLA ANTIBODY SURVEY IN SERA TAKEN DURING ETHIOPIAN EPIDEMIC
TCHABDRA AGORA

ILL PATIENTS	ILL PATIENTS WITHOUT FLAVIVIRUS ANTIBODY	ILL PATIENTS WITH EBOLA ANTIBODY
18	5	4

Table 8

HAEMORRHAGIC VIRUS ANTIBODY PREVALENCE IN NIGERIA (BENUE RIVER), 1965-66

VIRUS				
CONGO	RVF	EBOLA	LASSA	MARBURG
14%	5%	5%	10%	1.4%

Table 9

SUMMARY OF EBOLA ANTIBODY SURVEY IN SERA TAKEN DURING ETHIOPIAN EPIDEMIC

A. NON-EPIDEMIC AREA

TESTED 227 (AWASCH, ASSAB, BLUE NILE, SIDAMO-BORENA, OGADEN)

MONOTYPIC YF POSITIVES 11%

POLYTYPIC GROUP B 19%

EBOLA POSITIVES 21%

HOWEVER, 27 OF 48 EBOLA POSITIVES (56%) CAME FROM BLUE NILE.

WITHIN BLUE NILE, 13 OF 17 (76%) FROM ONE VILLAGE WERE POSITIVE.

IN CONTRAST, IN OGADEN, 1 OF 54 (0.02%) WAS POSITIVE.

Table 10

SUMMARY OF EBOLA ANTIBODY SURVEY IN SERA TAKEN DURING ETHIOPIAN EPIDEMIC

B. EPIDEMIC AREA

TESTED 50 (WOLLENGA, GAMBATTA, ILLUBABOR, KAFFA)

MONOTYPIC YF POSITIVES 4%

POLYTYPIC GROUP B 48%

EBOLA POSITIVES 22%

HOWEVER, IN TCHABORA AGORA (KAFFA) 38% WERE POSITIVE AS CONTRASTED

WITH GAMBATTA WHERE 10% WERE POSITIVE.

The breakdown of positives by region showed more positives in the Blue Nile and Valle deL'Aquache regions than in the Assab, Sidamo-Borena regions. (Only 4 sera were tested from Ogaden). However, within the Blue Nile region, 8 of 26 total positives (31%) come from two villages where each individual tested was positive. There were 25 villages sampled in the region. Similarly, in the Valle deL'Aquache, 8 of 14 total positives (57%) came from 2 villages. There were 12 villages sampled in this region. It appears from these preliminary data that there is a marked localization of virus activity within these broad geographic regions. Again, in the Sidamo-Borena region, 8 of 10 positives came from 3 villages of 25 sampled. Further testing was done with Ebola slides.

The results of HI tests during the Ethiopian YF epidemic have been summarized in Tables 1-3. In Kaffa province, 49% of the ill population had group B antibody, 13% group A, 26% specific for YF, 7% specific for Zika, and 11% of the population were non-diagnostic. Similar results were seen in the Village of Tchabora Agora. 75 patients were tested. 28 (37%) were YF positive. Of the 28, 18 were ill patients. However, there were 5 (28%) ill patients who had no group B antibody and never sero-converted to YF. These patients never developed antibody to any virus known at the time including a variety of bunyaviruses. Casals attempted to isolate an agent by inoculation of blood into mice, but was unsuccessful. In contrast, in the village of Gora, of 39 patients tested, 29 (74%) had group B antibody and of those, 19 (65%) were monotypic YF antibody positive.

These results and our positive CRELM reactions led us to explore the hypothesis that a concurrent outbreak of Ebola virus may have occurred during this YF epidemic.

Emphasis was given to study of sera taken in non-epidemic and epidemic areas during the Ethiopian epidemic. In the non-epidemic area, 227 sera were tested from Awasch, Assab, Blue Nile, Sidamo-Borena, and Ogaden. The results are given in Table 4. In this area, 11% of the sera were monotypic YF positives, 19% were polytypic group B positives, and 21% were Ebola positives. However, 27 of 48 Ebola positives (56%) came from Blue Nile. Within Blue Nile, 13 of 17 (76%) from one village were positive. In contrast, in Ogaden, 1 of 54 (0.02%) were positive.

In the epidemic area, 50 sera were tested from Wollenga, Gambatta, Illubabor, and Kaffa. Four percent were monotypic YF positives, 48% were polytypic group B positives, and 22% were Ebola positive. Again, distinct localization was noted since in Tchabora Agora (Kaffa), 38% were positive as contrasted with Gambatta where 10% were positive (Table 5).

The most convincing evidence for Ebola infection stems from study of ill patients within the epidemic region. In Tchabora Agora, there were sera from 18 ill patients available for study. 5 of these patients did not have flavivirus antibody. Of these five, 4 patients had Ebola virus antibody (type IgG). The single remaining patient had no IgG antibody, but did have antibody detectable with a fluorescein-labelled anti-human IgM conjugate (Table 6).

PROBLEMS IN INTERPRETING EBOLA IFA TEST RESULTS.

We are aware of two problems in interpreting our results. First, there is

no second confirmatory test currently in use. There are unexpected positive results in both the IFA and RIA. Many of these false positive reactions with only VP3 protein of Ebola.

SUMMARY AND CONCLUSION.

The geographic clustering which we have found supports the theory that an Ebola outbreak occurred concurrently with the YF outbreak. In surveys of other regions in equatorial Africa, we have never seen percentages as high as those observed during this study. In a study of haemorrhagic virus antibody prevalence in Nigeria (Benue River), 1965-1966, we found 5% Ebola positives (Table 7). The number of positives in this area is significantly higher.

As further evidence of specificity, approximately 250 sera collected recently from children in Ethiopia were surveyed for antibody and were found to be negative.

RETROSPECTIVE SEROSURVEY OF NIGERIA.

A retrospective study was done of sera collected in the early 1960's from the Benue river basin of Nigeria. The sera came from a WHO survey using randomly stratified serum sampling. Four hundred seventy serum samples were retrospectively examined for polyvalent (Congo, Rift Valley Fever virus, Ebola Sudan, Ebola Zaire, Lassa and Marburg) viral antibodies. One hundred ninety three were positive to the polyvalents, i.e., 41% positive. Monovalent antibodies were then tested for.

In the study for Congo virus antibody 116 of 193 (60%) of polyvalent positives were tested. Ten percent were positive of the 49 males tested (20%). For females, 22 were positive of the 67 tested (33%). A projected value of 14% of the total samples are estimated to be positive.

In the Rift Valley Fever virus study 158 of 193 (81%) of polyvalent positives were tested, 11 of 63 (17%) males were positive, and 14 of 95 females were positive (15%). A projected value of 5% of the total sample population was positive.

Ebola Sudan and Ebola Zaire data were merged. 113 of 193 polyvalent positives were tested (58%). Fourteen of 113 tested for Ebola (Zaire and Sudan) were positive (12%)/ Ebola Zaire monotypic, 7/113 (6%) and Ebola Sudan monotypic 37 (33%) were positive. A projected value of 19 percent of the total sample population was positive to Ebola virus.

In the Lassa virus antibody study, 190 of 193 (98%) of polyvalent positives were tested. Fifteen of 78 (19%) males and 31 of 112 (28%) females were positive. A projected value of 9.8% of the total sample population is positive.

In the Marburg virus antibody study, 166 of 193 (86%) of polyvalent positives were tested, 2 of 66 (3%) males and 5 of 100 (5%) females were positive for Marburg virus antibody. A projected value of 1.4 percent of the total sample population had Marburg antibody.

Table 11

Population Tested from Benue River Basin Nigeria 1965/66

A G E - IN YEARS

Sex	0-4	5-9	10-19	20-29	30-39	40-49	50-59	60+	Total
Male	42	57	42	29	23	7	9	8	217
Female	41	55	51	50	31	11	9	5	253
Percent of Total Population	18	24	20	13	11	4	4	3	

Table 12

Age and Sex Distribution of CRE₂LM Positives Benue River Basin, 1965/66

A G E - IN YEARS

Sex	0-4	5-9	10-19	20-29	30-39	40-49	50-59	60+	Total
Male	13/42	25/27	10/42	12/29	12/23	3/7	4/9	1/8	80/217
% +	31	44	24	41	52	43	44	13	37
Female	10/42	28/55	18/51	21/50	18/31	7/11	6/9	5/5	113/253
% +	24	51	35	42	58	64	67	100	45

% of population which is CRE₂LM positive = 41%

Table 13

Haemorrhagic Viruses in Nigeria (Benue River Basin)
An Ecologic Study (Retrospective) (1965/66)

	Tested on CRE ₂ LM	# Positive	% Positive
1A	1008	602	60
1B	694	438	63
1C	470	193	41

- 1A. Total number of sera samples tested.
- 1B. Total number of sera samples tested, having discarded
314 samples, for not having sampling areas on the
Masterlist.
- 1C. Finally, total number of sera samples used for analysis.

Table 14

CRE₂LM Positives Tested for RVFV Antibody
by Age and Sex, 1965/66

A G E - IN YEARS

Sex	0-4	5-9	10-19	20-29	30-39	40-49	50-59	60+	Total
Male	10	16	10	10	10	3	4	0	63
% of CRE ₂ LM Positives	76	64	100	83	83	100	100	0	79 (80)
Female	9	24	18	15	15	5	4	5	95
% of CRE ₂ LM Positives	90	86	100	71	83	71	67	100	85 (113)

Distribution of RVFV Antibody Positives by Age and Sex

A G E - IN YEARS

Sex	0-4	5-9	10-19	20-29	30-39	40-49	50-59	60+	Total
Male	2	0	2	3	2	0	2	0	11
%	20	0	20	30	20	0	50	0	17
Female	0	4	3	1	2	1	2	0	14
%	0	17	17	7	13	20	50	20	15

Table 15

CRE₂LM Positives Tested for Congo Virus Antibody
by Age and Sex, 1965/66

A G E - IN YEARS

Sex	0-4	5-9	10-19	20-29	30-39	40-49	50-59	60+	Total
Male	9	14	5	8	7	3	3	0	49
% of CRE ₂ LM Positives	69	56	50	67	58	100	75	0	61 (80)
Female	8	19	12	10	9	1	4	4	67
% of CRE ₂ LM Positives	80	68	67	48	50	14	67	80	59 (113)

Distribution of Congo Virus Antibody Positives by Age and Sex

A G E - IN YEARS

Sex	0-4	5-9	10-19	20-29	30-39	40-49	50-59	60+	Total
Male	1	4	0	1	3	0	2	0	11
%	11	29	0	13	43	0	67	0	22
Female	3	5	5	2	5	1	1	0	22
%	38	26	42	20	56	100	25	0	33

Table 16

CRE₂LM Positives Tested for Marburg Virus Antibody
by Age and Sex, 1965/66

A G E - IN YEARS

Sex	0-4	5-9	10-19	20-29	30-39	40-49	50-59	60+	Total
Male	12	20	9	9	8	3	4	1	66
% of CRE ₂ LM Positives	92	80	90	75	67	100	100	100	83
Female	8	26	15	20	18	3	5	4	100
% of CRE ₂ LM Positives	80	93	83	95	100	43	84	80	88

Distribution of Marburg Virus Antibody by Age and Sex

A G E - IN YEARS

Sex	0-4	5-9	10-19	20-29	30-39	40-49	50-59	60+	Total
Male	0	2	0	0	0	0	0	0	2
%	0	10	0	0	0	0	0	0	3.0
Female	0	2	2	1	0	0	0	0	5
%	0	7.6	13	5	0	0	0	0	5.0

Table 17

CRE₂LM Positives Tested for Lassa Virus Antibody
by Age and Sex, 1965/66

A G E - IN YEARS

Sex	0-4	5-9	10-19	20-29	30-39	40-49	50-59	60+	Total
Male	13/42	22/57	12/42	11/29	11/23	3/7	5/9	1/8	78
% of CRE ₂ LM Positives	31	39	29	38	48	43	56	13	
Female	10/41	26/55	18/51	21/50	19/31	7/11	6/9	5/5	112
% of CRE ₂ LM Positives	24	47	35	42	61	64	67	100	

Distribution of Lassa Virus Antibody by Age and Sex

A G E - IN YEARS

Sex	0-4	5-9	10-19	20-29	30-39	40-49	50-59	60+	Total
Male	2	5	1	2	3	1	1	0	15
%	15	23	8	18	18	33	20	0	15/78 = 19
Female	2	6	5	5	5	3	3	2	31
%	20	23	28	24	26	43	50	40	31/112 = 28

Table 18

CRE₂LM Positives Tested for Ebola Virus Antibody
(Zaire and Sudan)
by Age and Sex, 1965/66

Sex	A G E - IN YEARS								Total
	0-4	5-9	10-19	20-29	30-39	40-49	50-59	60+	
Male	13	25	10	11	11	2	3	1	62
% of CRE ₂ LM Positives	63	56	100	92	92	67	75	100	78 (80)
Female	7	17	10	14	10	4	5	2	69
% of CRE ₂ LM Positives	70	70	56	67	56	58	83	40	61 (113)

Distribution of Ebola Virus Antibody by Age and Sex

Sex	A G E - IN YEARS								Total
	0-4	5-9	10-19	20-29	30-39	40-49	50-59	60+	
Zaire	3	4	0	1	3	1	0	0	12
Male	3	1	3	4	4	0	1	2	18
Female									
Sudan									
Male	4	4	3	1	3	0	3	0	18
Female	0	8	4	3	5	2	1	0	23
Z and S									
Male	2	1	1	1	0	0	2	1	8
Female	0	1	1	4	1	0	1	0	8
% Male	15	4	10	9	0	0	67	100	
% Female	0	6	10	29	10	0	20	0	

Table 19

Summary of Ebola Antibody Studies

	#	%
Tested on Zaire and Sudan	113	58% (193)
Negative on Both	55	49%
Positive on Both	14	12%
Zaire Monotypic	7	6%
Sudan Monotypic	37	33%

Summary of Approximate Antibody Prevalences

	V I R U S				
	Zongo	RVF	Ebola	Lassa	Marburg
Prevalence	14%	5%	19%	9.8%	1.4%

Table 20

Summary of Virus Antibody Studies in Benue River
Basin, 1965/66

	Congo	RVFV	Ebola Zaire	Ebola Sudan	Lassa	Marburg
Total Tested	116/93	158/193	131/193		190/193	166/193
Males	49	63	62		78	66
Females	67	95	69		112	100
% of CRE ₂ LM Positive	60	81	68		98	86
Positive by Sex						
Males	10/49	11/63	30/62		15/78	2/66
%	20	17	48		19	3
Females	22/67	14/95	41/69		31/112	5/100
%	33	15	59		28	5
Projected % of Sample Positive	14	5	19		9.8	1.4
p > 0.05, male/female difference not statistically significant						

Sex differences were not statistically significant (0.05); sampling variation is a likely explanation of the differences seen. There were no geographic occupational, rural/urban differences, as all of these factors were the same to the area studied.

Evidence presented above suggests that Lassa virus was present in the Benue river basin prior to its official recognition as a disease entity.

DEVELOPMENT OF THE ELISA TEST FOR CCHF VIRUSES.

We have used detected CCHF virus infected cells by ELISA. This system offers promise of a safe way for titration of infectious virus and for detection of antibody. This system can be used until development of monoclonal and anti-idiotypic reagents replaces it. In developing these reagents, this system will be useful.

1. Brief Protocol Outline

CER cells are seeded at 5×10^5 cells per well. On the following day, alternate rows of cells are infected with virus. Plates are incubated in a humidified chamber within a 5% CO₂, 37°C incubator for the required period of time, at least 4 days. On the required day, to wells containing 200 ul, is added 100 ul of 10% neutral buffered formalin, (final concentration, 3%). The plates are held for at least 24 hrs before further processing. After 24 hrs, the medium is poured off and the plates are washed twice. Two-hundred ul. of blocking buffer is added for 1 hr at 37°C. The plates are washed twice, and diluted antibody is added to the wells for 1 hr. After washing 3x, 100 ul of the anti-species antibody is added for 1 hr. Plates are washed 5x, and the substrate is added. In our tests, the conjugate is peroxidase and the substrate is ABTS. After color development, the reaction is stopped on ice and the plates are read at 414 nm.

2. Virus Enumeration by ELISA

CCHF strain 10200 was titrated in CER cells and plates were harvested each day. Background was derived from mock infected wells adjacent to each virus infected well. The virus titer on days 4, 5, and 6 were $10^{5.2}$, $10^{5.3}$, and $10^{4.7}$ respectively. Other virus strains were similarly titrated in CER cells and titers were derived as described above.

3. Antibody Detection by ELISA

Using CCHF virus infected plates, antibody to each strain was titrated on homologous and heterologous virus infected plates. Strains differences were not observed by this method. OD readings for control wells ranged from 0.27 to 0.32.

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